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MIXED FUNCTION OXYGENASE ACTIVITY IN BLUE CRAB, *CALLINECTES SAPIDUS*: TISSUE DISTRIBUTION AND CORRELATION WITH CHANGES DURING MOLTING AND DEVELOPMENT

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A group of microsomal enzymes, referred to as mixed function oxygenases, are responsible for the metabolic modification of many foreign compounds in animals. Due to the introduction of industrial wastes and fossil fuels into the ocean, the fate and effects of foreign compounds in marine animals are of interest. Uptake and discharge of petroleum hydrocarbons at nontoxic levels have been examined in several marine species including fish (Lee, Sauerheber and Dobbs, 1972b), crabs (Corner, Kilvington and O'Hara, 1973; Lee, Ryan and Neuhauser, 1976), shrimp (Anderson, Neff, Cox, Tatem and Hightower, 1974), mussels (Lee, Sauerheber and Benson, 1972a), clams (Neff and Anderson, 1975) and oysters (Stegeman and Teal, 1973; Stegeman, 1974). Hydrocarbons may be eliminated from marine animals either unmodified or as polar metabolites (Stegeman and Teal, 1973; Corner *et al.*, 1973). Arylhydrocarbon hydroxylase, a mixed function oxygenase, is involved in the hydroxylation of the aromatic ring, an early step in the metabolism of aromatic hydrocarbons. This enzyme has been characterized in mammals (Conney, Miller and Miller, 1957; Nebert and Gelboin, 1968; Gelboin, 1972; Owens and Nebert, 1976; Atlas and Nebert, 1976), fish liver (Pedersen, Herschberger and Juchau, 1974; Payne and Penrose, 1975; Philpot, James and Bend, 1976) and is present in green gland of fresh water crayfish (Khan, Coello, Khan and Pinto, 1972) and blue crab (Lee, Furlong and Singer, 1977). The occurrence of enzyme activity in several tissues of the blue crab, *Callinectes sapidus*, with varying levels of activity dependent on both sex and stage of development, is reported in this study.

Materials and Methods

Blue crabs, *Callinectes sapidus*, were collected by trawling or trapping in estuaries of coastal Georgia (U.S.A.) between May and September when the median water temperature is 28° C. The crabs were maintained in tanks receiving flowing filtered sea water of 20‰ salinity (White, Stickney, Miller and Knight, 1973) and were segregated according to sex and size. Those specimens in the final stages prior to ecdysis or for special treatment (diet or eyestalk removal) were placed in individual aerated seawater aquaria. All crabs were fed three times weekly with frozen shrimp.

Internal tissues were located, identified and dissected using references to the general biology of the blue crab (Cronin, 1947; Pyle and Cronin, 1950; Waterman and Chace, 1960; Passano, 1960; Bollenbacher, Flechner and O'Connor, 1972; Carlisle and Connick, 1973). Stages of the molting cycle were classified using the guidelines summarized by Passano (1960).

Crude homogenates were prepared from dissected tissues in 0.15 M KCl buffered with 0.05 M Tris, pH 7.4, using a Potter-Elvehjem homogenizer. Cell debris and nuclei were removed by centrifugation at $700 \times g$ for 10 minutes at 4° C. Supernatants were maintained at 4° C until assay of enzyme activity.

Arylhydrocarbon hydroxylase was assayed by the method of Wattenberg, Leong and Strand (1962), with the modifications described by Nebert and Gelboin (1968). The assay mixture contained 0.6 μmoles NADPH (Sigma Chemical Co.), 3 μmoles $MgCl_2$, 0.01 μmoles benzo(a)pyrene (Sigma Chemical Co.) and crude tissue homogenates (0–4 mg protein) in a total volume of one ml. The mixture was incubated at 28° C for 30 minutes and stopped by the addition of 1 ml cold acetone and 3 ml hexane. Two ml portions of the resulting organic phase were extracted with 4 ml normal NaOH and fluorescence of products recorded with activation of 396 nm and emission at 522 nm (Turner Model 430). Assays were done in triplicate with a blank containing homogenate boiled for 30 seconds prior to addition of substrates. The assay was determined to be linear with both time and protein under these conditions in green gland homogenates. One unit of enzyme activity was defined as the fluorescence produced in a 60 minute incubation at 28° C equivalent to the fluorescence of 1×10^{-12} moles of 3-hydroxybenzo(a)pyrene.

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as the standard.

Results

Large male crabs of 14 to 17 cm in carapace width were judged mature. Specific activities of crude homogenates were determined in ten tissues (Table IA) as outlined in methods. Activity was detected in only nine tissues, the cardiac muscle apparently lacking activity. In blood, gill, reproductive tissues, eyestalk, and hepatopancreas, median values were less than 150 units activity per mg protein. Higher activity was seen in green gland homogenates (450 ± 270 units per mg protein). However, the highest activity in the male was found in the pyloric stomach which had 1400 ± 170 units per mg protein.

Mature female crabs were easily differentiated by the broadened abdomen (apron) on the ventral side of the body. Upon internal inspection, only mature females with developed ovaries were used to determine specific activities (Table IB). Specific activities in blood, gill, reproductive tissues, eyestalk, cardiac muscle, and hepatopancreas were about the same as in the male. The enzyme activity of pyloric stomach was slightly higher than that found in males, and enzyme levels of the female green gland were almost two orders of magnitude higher than found in the males.

Female specimens showed no decrease in green gland activity one day after removal of eyestalks. However, females which survived four and six days after eyestalk removal showed a decrease in green gland activity. After six days, the activity was approximately one half of control activity. The green glands are located near the base of the eyestalks and may be affected by a hormone secreting gland (designated x-gland) located within the eyestalk.

Growth in crustaceans requires a series of molts. In the female blue crab there are approximately 20 postlarval molts prior to maturity (Von Engel, 1958), with each molt resulting in an increase in carapace width of about one third. The final

TABLE I

Specific activities of arylhydrocarbon hydroxylase in various tissues of blue crabs. One unit of enzyme activity equals the fluorescence equivalent to 1 × 10^{-12} moles of 3-hydroxybenzo(a)pyrene produced in a 60 minute incubation of 28° C. Specific activity values are the mean ± standard deviation with the number of assays given in parentheses.

Tissue	Total activity (units)	Specific activity (units/mg protein)	
A. Mature Males			
green gland	950	450 ± 270	(8)
hepatopancreas	560	10 ± 12	(13)
testes	1,300	150 ± 110	(13)
median vas deferens	300	18 ± 13	(3)
proximal vas deferens	1,500	15 ± 15	(3)
gill	6,900	140 ± 80	(12)
blood	5,000	3.1 ± 1.1	(2)
pyloric stomach	20,000	1,400 ± 170	(4)
eyestalk	43	140 ± 26	(3)
heart	–0–	–0–	(1)
B. Mature Females			
green gland	109,000	34,000 ± 2,700	(7)
hepatopancreas	1,400	9.4 ± 6.5	(6)
ovary	170	7.7 ± 7.6	(4)
seminal receptacle	800	97 ± 91	(5)
gill	1,900	140 ± 124	(3)
blood	3,000	2.6	(1)
pyloric stomach	49,000	2,500 ± 910	(6)
eyestalk	90	87	(1)
heart	500	21	(1)

molt produces a mature female ready for breeding. Upon reaching maturity, evidence indicates that the female does not molt successfully again even though she may live up to three years. Arylhydrocarbon hydroxylase in green glands was measured in two general classes of immature female blue crabs. The first was judged to be in cycles prior to the final maturing molt. Enzyme activities of this group were compared according to carapace width. Crabs, 5 to 5.5 cm in size, molt to produce individuals of about 7 cm which, in turn, molt to produce 9 cm specimens. When plotted (Fig. 1), seven immature female crabs from these three molt cycles gave a linear correlation between green gland activity and carapace width ranging from 3400 to 29,000 units per mg protein. None of the crabs showed evidence, either external or internal, of being near ecdysis.

The second class of immature females was in the final molt cycle but not yet in the enforced fast which occurs three to seven days prior to ecdysis. The intermolt prior to this cycle extends up to seven months (Truitt, 1939). Specimens in this group were difficult to judge externally and internally, and enzyme levels of arylhydrocarbon hydroxylase varied greatly. Five specimens of the same carapace size (±0.5 cm) were graded stages 1 to 5 as the color of the apron darkened. Stages 1 through 4 were nonfasting and did not have molting rings on the walking legs. Stage 5 had the darkest apron and was nonfasting but did have pink molting rings

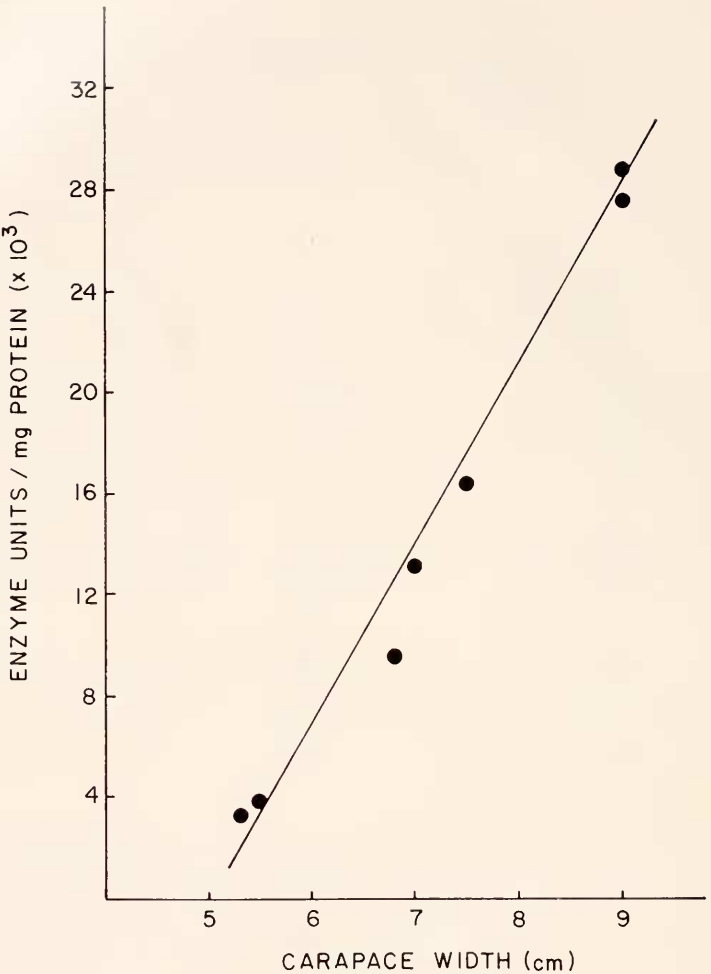

FIGURE 1. Increase in arylhydrocarbon hydroxylase activity in green gland of immature female blue crabs. Specimens chosen were intermolt from three different molt cycles prior to the final molt for maturation.

clearly visible on the walking legs. In general, as the color of the immature apron darkened, the enzyme level of green gland increased from 4,000 units/mg protein in stage one to 31,000 units/mg protein in stage four. Stage five, which was entering the later stages of proecdysis, had a green gland activity of 12,000 units/mg protein. Two females were examined which were in a fasting state which immediately precedes shedding (Table II). Both had less than one-tenth normal adult enzyme activity. Females taken during the first four hours after ecdysis showed enzyme activity levels of 7800 ± 4000 units per mg protein. Hardening of the new shell begins four or five hours after shedding and continues for about two days. Individuals which were ten hours and twenty hours postecdysis showed

activities of 24,000 and 26,000 enzyme units per mg protein, respectively. After 60 hours, the enzyme had reached levels of 35,000 enzyme units per mg protein, well within the range of activity found in mature adults. Other tissues assayed included hepatopancreas, gill, and stomach. Excluding green gland, no significant variation from adult levels of arylhydrocarbon hydroxylase was found in tissues of female immature crabs.

One small *Callinectes sapidus* male (10.5 cm carapace width) was found, which upon internal inspection, was judged immature. Green gland activity measured 630 units per mg protein. This is within the range of the observed values in mature males. Other tissues measured were not significantly different in arylhydrocarbon hydroxylase from that found in mature males.

Previous reports have not noted significant activity in stomach tissue as was seen here in both mature male and female blue crabs (Table I). A large male crab was carefully dissected and fluid drawn by needle from the unruptured pyloric stomach. The stomach was then removed, its contents discarded, and the tissue carefully washed with buffer. The juice from the stomach had no detectable activity and when the fluid was added to the *in vitro* assay of stomach homogenate, no inhibition was observed. The effect of starvation on enzyme activity of the stomach was examined. Mature females were starved up to six days and enzyme activity determined daily. During this time no decrease in activity occurred.

Discussion

In this study, very low levels of the mixed function oxygenase, arylhydrocarbon hydroxylase, were found in *in vitro* assay of all tissues examined of *Callinectes sapidus* except the stomach and green gland. Recently, several tissues of the fiddler crab were surveyed for mixed function oxygenase using the aldrin epoxidation reaction (Burns, 1976). In agreement with our observations, the green gland had the highest activity, while in hepatopancreas much lower activities were noted. In contrast, the *in vivo* data of Lee *et al.* (1976) concluded that the hepatopancreas is the site of hydrocarbon metabolism in the blue crab. Pohl, Bend, Guarino and Fouts (1974) postulated that digestive fluids produced in the hepatopancreas inhibited the *in vitro* assay of the enzyme.

In stomach tissue, significant levels of arylhydrocarbon hydroxylase were found.

Table II

Arylhydrocarbon hydroxylase in the female green gland just prior and after the final molt. Specific activity values are the mean ± standard deviation with the number of assays given in parentheses.

Stage	Time	Specific activity (units/mg protein)
Proecdysis		
fasting	3–7 days	2,000 ± 1,000 (2)
Postecdysis		
soft shell	0–4 hours	7,800 ± 4,400 (3)
paper shell	10–20 hours	25,000 ± 1,300 (2)
hard shell	2–14 days	35,000 ± 1,000 (2)

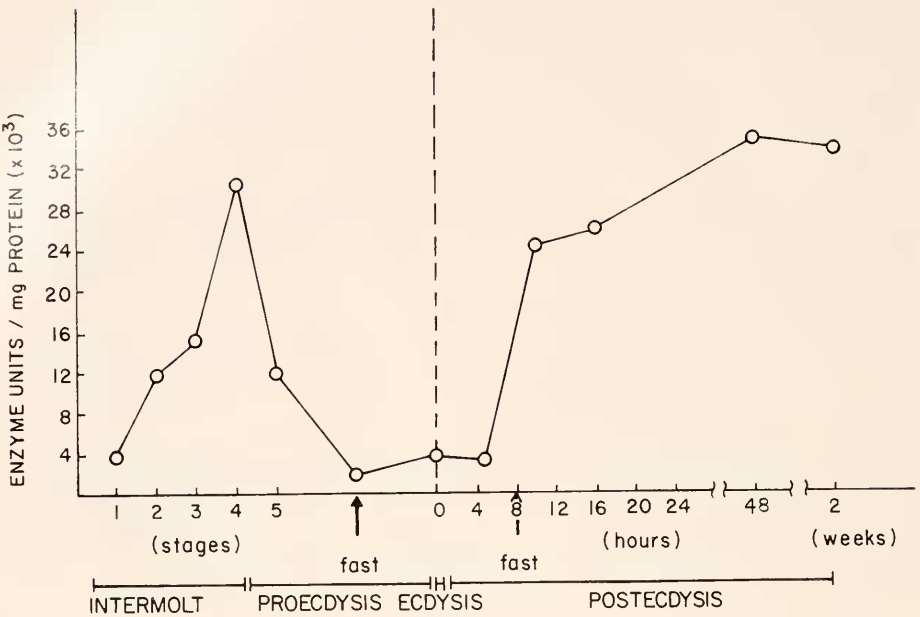

FIGURE 2. Fluctuation of arylhydrocarbon hydroxylase during molting in the green gland of the female blue crab. Intermolt stages were arbitrarily judged by darkening abdomen color. Proecdysis was judged by appearance of molting rings followed by a fast beginning 3–7 days prior to ecdysis. Postecdysis measured from moment when newly molted crab was free of old carapace.

This was unexpected, since the stomach is a chitinous structure and is shed at each molt. The activity was not due to any digestive fluid or parasites inside the stomach but rather was present in the washed tissue. In the male, the stomach accounted for most of the total activity. In experiments where crabs were fed a single dose of radiolabeled hydrocarbons, significant amounts of metabolites produced after one day were found in the stomach, whereas the hepatopancreas and blood contained more metabolites at longer time periods (Lee *et al.*, 1976). Additional experiments were not reported to remove the possibility that metabolites found in the stomach during *in vivo* studies were perhaps due to bacteria or other parasites. It would appear that the stomach, though not the primary site of metabolism, participates in the metabolism of aromatic hydrocarbons which are taken up from food.

The specific activity of arylhydrocarbon hydroxylase in green gland of egg bearing females was the highest found. Careful dissection of tissue eliminated possible contamination of green gland homogenates by tissue of the x-gland from within the eyestalk or by the y-organ (mandibular organ). However, a gland reported by Carlisle and Connick (1973) in the antennary segment of crayfish was not excluded from the green gland homogenates. In fact, some confusion exists as to whether or not these are indeed different tissues. Arylhydrocarbon hydroxylase activity in green gland increased in females as they progressed through the postlarval molts

prior to maturity. Molting occurs during the growth of all arthropods. In insects, microsomal epoxidation activity fluctuates during development (Perry and Buckner, 1970; Yu and Terriere, 1971). Using data presented in the text and Table II, a summary diagram of arylhydrocarbon hydroxylase changes in crabs during development can be produced (Fig. 2) which appears quite similar to the changes in microsomal epoxidation activity in insects during their molting cycles (Wilkinson and Brattsten, 1972). In the female blue crab, molting rings became visible on the walking legs as the time of ecdysis approached, followed by an enforced fast. The enzyme levels which increased during intermolt began to fall and at ecdysis were at a very low level. Following ecdysis, the enzyme levels again rose as the newly molted crab proceeded through the soft and semisoft (paper) stages to a hardened exoskeleton. The crab did not begin to feed until the shell was completely hardened. Though males were not examined in this manner, it could be expected that enzyme levels would change in a similar pattern during molting. However, since the intermolt enzyme levels of males are much lower than females, the variation would not be as marked.

With live crabs, green glands showed no build up of any radiolabeled hydrocarbon and the presence of only polar metabolites (Lee *et al.*, 1976) as would be expected of an organ with mainly excretory functions. Why, then, is enzyme activity so high in a tissue with only known excretory functions and which in previous experiments has not been shown of *in vivo* importance? Fluctuations in enzyme activity during the molt cycles suggested a hormonal influence. This was further supported by the decrease in activity of green gland soon after removal of the eyestalks, which eliminated a supply of hormones produced by the x-glands.

Speculation as to the answer to the above question without further investigation is risky; however, green gland levels of arylhydrocarbon hydroxylase and molting hormones can be correlated in the following manner. Molting in crustaceans is controlled by a group of steroid hormones called ecdysones produced by the y-organs (Passano, 1960; Goad, 1976). During intermolt, a molt inhibiting hormone produced by the x-gland is present which suppresses the y-organ (Goad, 1976). Ecdysones were measured in *Callinectes sapidus* during three stages of the molt (Faux, Horn, Middleton, Fales and Lowe, 1969). The hormones were lowest in intermolt, higher in the proecdysis and highest just after ecdysis when the crab was soft. Arylhydrocarbon hydroxylase levels were inversely related to the ecdysones, were highest in intermolt, falling during proecdysis, and were lowest just after the molt when the crab was soft. Immediately following ecdysis, levels of arylhydrocarbon hydroxylase increased rapidly when the levels of ecdysones undergo an opposing decrease.

In addition to modification of foreign compounds, the mixed function oxygenases also function in the metabolism of bile acids, fatty acids and steroid hormones (Conney and Klutch, 1963; Greim, Trulzsch, Czygan, Hutterer, Schnaffner, Popper, Copper and Rosenthal, 1973; Czygan, Greim, Trulzsch, Rudick, Hutterer, Schaffner, Popper, Rosenthal and Cooper, 1974). In hepatic tissues, steroids are the likely natural substrates of the mixed function oxidases (Conney, 1967), and the interactions of chlorinated hydrocarbons with steroid hormones has been recently reviewed (Kupfer and Bulger, 1976). The green gland as an excretory organ monitors the blood and controls ion levels of the hemolymph (Robertson,

1960). It appears from our results that the green gland may also function in metabolism of steroid hormones. Crustecdysone, a steroid molting hormone, is a 20-hydroxy derivative of a cholesterol-like precursor which has been found by Carlisle and Connick (1973) to be present only in a gland of the antennary segment of crayfish. Conversion of dietary derived cholesterol to crustecdysone would require a 20-cholesterol hydroxylase activity. The enzyme activity of green gland attributed to arylhydrocarbon hydroxylase may be a similar steroid hydroxylase.

The biological effects of foreign compounds on marine life are not well understood. In addition to acute toxicity, reduced growth and reproduction pose a threat to the survival of marine organisms. If mixed function oxygenases contribute to the control of molting hormone levels, then the presence of aromatic hydrocarbons acting as substrates for these enzymes could alter the rate at which the crab passes through the early molts. Several studies with chlorinated hydrocarbons, a second group of foreign compounds, have shown that juvenile decapods are more sensitive than adults (Epifanio, 1971; Nimmo, Blackman, Wilson and Forester, 1971). Increasing concentrations of methoxychlor prolonged larval development to the first juvenile stage of the blue crab, *Callinectes sapidus*, the mud crab, *Rhithropanopeus harrisii* (Bookhout, Costlow and Monroe, 1976), and the dungeness crab, *Cancer magister* (Armstrong, Buchanan, Mallon, Caldwell and Millemann, 1976).

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Summary

We have presented results of assay of various internal tissues of blue crabs, *Callinectes sapidus*, finding high activity of arylhydrocarbon hydroxylase in the stomach and green gland. Also we have noted that the green gland activity of this enzyme varies at different stages of maturity and molt cycle in a manner similar to insects. We have speculated as to why an excretory organ should have such high activity and undergo such fluctuations in activity.

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